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(71) Applicant *(for all designated States except US)*: SURFACE ACTIVE LIMITED [GB/GB]; Ground floor, 11/12 Queen Square, Bristol BS1 4NT (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: RANDLE, Beverley, Jane [GB/GB]; 19 Upper Belgrave Road, Bristol BS8 2XL (GB).

(74) Agent: DEAN, J., P.; Withers & Rogers, 4 Dyer's Buildings, Holborn, London EC1N 2JT (GB).

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(57) Abstract

The invention provides a diagnostic method for determining the presence, or absence of an antigen in a sample, the method comprising contacting the sample with a multispecific antibody having first and second binding sites for binding the antigen and a detectable entity respectively, binding of the detectable entity to the antibody inactivating the detectable entity in the presence of an analogue of the antigen, wherein binding of the analogue of the antigen to the antibody results in release of the detectable entity in a detectable form from the antibody but binding of the antigen to the antibody does not cause such release or causes significantly lower levels of release. Preferably, the antigen is a subunit of inhibin.

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DIAGNOSTIC METHOD

This invention relates to a diagnostic method and is particularly, though not exclusively, concerned with an assay for relatively small antigens such as inhibin.

Inhibin is a non-steroidal gonadal glycoprotein central in the regulation of pituitary Follicle Stimulating Hormone (FSH) secretion. Inhibin inhibits secretion of FSH by the pituitary (reviewed in Ying SY (1989), *J. Steroid.* **33**, 705). Inhibin consequently has a key role in control of ovulation and sperm production. Inhibin and associated glycoprotein hormones have biological effects outside the reproductive system, with growth factor properties in the central nervous system and the immune response. Biotechnological applications of inhibin are widespread. In man, serum inhibin is a highly specific indicator of fertility in *In Vitro* Fertilisation (IVF), and other clinical situations. Inhibin is also proving an excellent marker for management of certain forms of ovarian cancer. Experimental uses of inhibin biotechnology in animal husbandry have included induction of ovulation, extension of breeding season, and assessment of reproductive capacity.

The extensive commercial applications of inhibin biotechnology have yet to be realised as progress has been severely hampered by lack of an assay for bioactive inhibin and a source of the purified molecule.

Purification of inhibin in 1985 (Robertson DM *et al* (1985), *Biochem Biophys. Res. Commun.* **126**, 220) led to a partial amino acid sequence and determination of the structure of the molecule (Mason AJ *et al* (1985), *Nature* **318**,659). The two forms of 32 kiloDalton (kD) inhibin, A and B, consist of a common α subunit, 18 kD, and similar, but distinguishable, β subunits, β A and β B, 14kD. The α subunit is joined to either β subunit by disulphide linkage to give bioactive inhibin (Finlay JK *et al* (1987), *J Reprod Fertil* **80**, 455). The biological activity of inhibin is mediated by the β subunit.

Secretion of the α subunit monomer has been detected, but the glycoprotein lacks the

biological activity characteristic of inhibin (Knight PG *et al* (1989), *J. Mol. Endocrinol.* **2**, 189). Activin is the secreted dimer of the β subunit and, in contrast to inhibin, enhances FSH secretion, despite the structural similarity to inhibin (Ling N *et al* 1986), *Nature* **321**, 779). The β subunit has strong homology for Transforming Growth Factor (TGF). However, secretion of free β subunit monomers has yet to be discovered.

Production of inhibin is regulated mainly by FSH. Inhibin secretion, in response to FSH from the pituitary, originates primarily from the granulosa cells of the ovary and the Sertoli cells of the testis. Serum inhibin levels in woman rise to maximum in the mid-luteal phase of the menstrual cycle and decline prior to menstruation. In pregnancy the luteal phase does not occur, and levels increase progressively, suggesting inhibin is also produced by the corpus luteum and the placenta (Abe Y *et al* (1990), *J. Clin. Endocrinol. Metab* **71**, 133). In man, inhibin is secreted in well-defined pulses from the testis, coincident with testosterone release (Winters SJ (1990), *J Clin Endocrinol Metab* **70**, 548). Serum inhibin levels rise throughout normal male and female puberty, where gonadal inhibin production is stimulated by increasing FSH levels (Burger HG *et al* (1988), *J Clin Endocrinol Metab* **67**, 689).

The biological effects of inhibin have been demonstrated by *in vivo* immunisation against the molecule. Antibody blocking of inhibin secretion in ewes leads to raised serum FSH and, in some studies, to an increase in ovulation (Forage G *et al* (1987), *J. Endocrinol.* **11**, PR1). In the male rat, reduction of serum inhibin by immunisation results in decreased sperm production and a significant fall in fertility (Vanage GR *et al* (1990), *Mol. Reprod. Dev.* **25**, 227). With the reciprocal interaction between FSH and inhibin, inhibin has applications as a contraceptive in the male, and as an agent for treatment of infertility in the female.

Both inhibin and activin have biological effects outside the reproductive system, having functions as growth factors, and mRNA for the glycoproteins has been detected in other tissues. Inhibin inhibits the synthesis of haemoglobin in human bone marrow whilst activin stimulates haemoglobin production (Yu J *et al* (1987), *Nature* **330**, 765). Activin is also

found to control oxytocin secretion in central neural pathways (Sawchenko PE *et al*, *Nature* 334, 615). In common with TGFβ, both inhibin and activin regulate T cell differentiation *in vitro*, suggesting a role for these glycoprotein hormones in control of the immune response (Hedger MP *et al* (1989), *Mol Cell Endocrinol* 61, 133).

Serum inhibin levels are elevated in women suffering from ovarian granulosa cell tumours. Inhibin levels are raised months before overt tumour recurrence, at times when the clinical tumour marker of oestradiol levels is still normal (Lappohn RE *et al* (1989), *N. Engl. J. Med.* 321, 790). Inhibin may also be a useful marker for hydatidiform mole, where raised inhibin levels after surgery indicate residual trophoblastic tumour disease (Yohkaichiya T *et al* (1989), *Br. Med. J.* 298, 1684).

Inhibin biotechnology has both immediate and far reaching applications in medicine and animal husbandry. At present, medical use of inhibin biotechnology relate to measurement of serum inhibin for clinical evaluation of fertility and for tumour diagnosis.

Measurement of serum inhibin has proved useful in assessment of reproductive capacity in a variety of clinical situations. Restoration of normal sperm production in treatment of hypogonadism is indicated by increased serum inhibin levels, in excess of normal values (Tsatsoulis A *et al* (1990), *Horm Res* 33, 18). In women, decreasing serum inhibin levels are an early index of declining ovarian function with advancing age (Hughes EG *et al* (1990), *J. Clin. Endocrinol. Metab.* 70, 358). In IVF Programmes, serum inhibin is proving a valid index of follicle maturation prior to use of the ovum for *in viro* fertilisation (Tsuchiya K *et al* (1989), *Fertil. Steril.* 52, 88).

Measurement of serum inhibin also has major use in treatment of women suffering from granulosa cell tumours. Granulosa cell tumours account for 10% of ovarian cancer cases and arise from specialised cells in the ovarian stroma which secrete inhibin. Primary treatment is surgical, and 80% of patients die of recurrent disease. Serum inhibin has proved an excellent indicator of the size of the primary tumour and of early disease recurrence, prior to overt clinical evidence. As the recurrent disease responds well to

combined chemotherapy, a serum inhibin assay has significant application in clinical management of women suffering from granulosa cell tumours.

A second generation of medical applications is indicated, related to alteration of circulating inhibin levels. Selective inhibition of FSH secretion in the male, by increasing serum inhibin levels, has potential for male contraception by reducing sperm production (Frick J and Aulizky W (1988), *Hum Reprod* 3, 147). In contrast, in woman, artificially-reduced inhibin levels can potentially increase FSH secretion and improve fertility. Treatment with anti-inhibin agents has great potential for IVF Programmes, where conventional fertility drugs have a high risk of multiple pregnancy.

The biotechnology of inhibin is already well advanced in animal husbandry. Manipulation of serum inhibin levels by passive and active immunisation against inhibin (Mann GE *et al* (1990), *J. Endocrinol.* **125**, 417 and Forage G *et al* (1987), *J. Endocrinol.* **114**, PR1) has led to increased ovulation rates in sheep, and by active immunisation , in cattle (Price CA *et al* (1987), *J. Reprod. Fertil.* **81**, 161). Puberty in ewe lambs can be advanced by active immunisation against inhibin (Al-Obaidi SA *et al* (1987), *J. Reprod. Fertil.* **81**, 403), and the breeding season of rams can be delayed, augmented and extended by anti-inhibin immunisation (Voglmayr JK *et al* (1990), *Biol. Reprod.* **42**, 81).

Full realisation of the potential applications of inhibin biotechnology is being hampered by lack of a purified inhibin source and by the inability to measure bioactive inhibin. The two problems are closely related, as lack of a purified source of inhibin has prevented development of the immunological reagents essential for inhibin assay design.

Production of a source of recombinant bioactive inhibin has proved difficult. Amino acid sequences of inhibin and activin have been deduced from cDNA and genomic clones (Mason AJ *et al* (1985), *Nature* **318**, 659 and Mason AJ *et al* (1989), *Mol. Endocrinol.* **3**, 1352). Recombinant inhibin has reduced biological activity, as glycosylation of both subunits, and associated subunit assembly, is necessary for full biological activity (Bremner WJ (1989), *N Engl J. Med.* **121**, 826). Recent studies indicate that pro-regions not present in activin

or TGFβ, are essential for folding, disulphide bond formation and export of the dimeric protein (Gray AM and Mason AJ (1990), *Science* **247**, 1328). These pro-regions must also therefore be present in isolated cDNA clones for production of recombinant inhibin. The first biologically-active recombinant inhibin A was subsequently reported in mid 1990 by Biotech Australia Ltd (Tierney ML *et al* (1990), *Endocrinology* **126**, 3268).

A source of recombinant inhibin will undoubtedly aid assay development, where traditional biochemical isolation of inhibin yields only 3% recovery, despite a 2,200 fold purification (Knight PG *et al* (1990), *Domest. Anim. Endocrinol* **7**, 299).

Inhibin bioactivity was first measured by a bioassay dependent on suppression of pituitary FSH secretion *in vivo* in ovariectomised ewes (Findlay JK *et al* (1987), *J. Reprod. Fertil.* **80**, 455) or *in vitro* by culture of rat pituitary cells (Lee VW *et al* (1987), *Aust. J. Biol. Sci.* **40**, 105). *In vivo* methods proved imprecise, insensitive and necessitated use of a large number of animals. In contrast, *in vitro* bioassay was quantitative but time-consuming, requiring two days for completion.

Development of antisera to bovine follicular fluid extracts permitted design of the first radioimmunoassay, RIA, for inhibin (McLachlan RI *et al* (1987), *J. Clin. Endocrinol. Metab.* **65**, 954). The heterologous RIA did not detect related glycoproteins of activin A or TGFβ, in comparison to a sheep pituitary cell bioassay (Robertson DM *et al* (1988), *J. Clin. Endocrinol. Metab.* **67**, 438). This lack of cross reactivity has since been found to result from the epitope specificity of the RIA being entirely localised to the α subunit of inhibin (Schneyer AL *et al* (1990), *J. Clin. Endocrinol. Metab.* **70**, 1208). The validity of the existing RIA may be questioned, as α subunit monomers of inhibin are secreted into human serum (Knight PG *et al* (1989), *J. Mol. Endocrinol.* **2**, 189. These monomers will be detected as bioactive dimeric α-β linked inhibin, giving a false positive result.

The first two site immunoassay for inhibin was reported at the end of 1990 by Medgenix Diagnostics, Liege, Belgium. The assay relies on capture and detection of two epitopes on the α subunit of inhibin. Assay inaccuracy, resulting from cross reaction to free α

monomer, is as yet unreported (Eliard P *et al* (1990), *Inhibin: Clinical Investigations. Brussels, Sept 1990)* but is believed to be a problem.

In our co-pending International patent application No. PCT/GB92/00769, the disclosure of which is incorporated herein by way of reference, we describe a novel assay system involving a bispecific antibody having closely adjacent binding sites for the analyte of interest and a entity such as an enzyme which is readily detectable when not bound to the bispecific antibody. For example, an enzyme active site may be blocked when the enzyme is bound to the antibody. The system is arranged such that binding of the analyte to the respective binding site of the antibody causes release of the detectable entity, as a result of steric interaction between the analyte and the entity.

Certain relatively small analytes such as inhibin may not readily be detected by such a system because their size reduces the steric interaction, even with a relatively large detectable enzyme.

It is an object of the present invention to provide an assay system for relatively small molecules such as inhibin.

According to one aspect of the invention there is provided a diagnostic method for determining the presence, or absence of an antigen in a sample, the method comprising contacting the sample with a multispecific antibody having first and second binding sites for binding the antigen and a detectable entity respectively, binding of the detectable entity to the antibody inactivating the detectable entity in the presence of an analogue of the antigen wherein binding of the analogue of the antigen to the antibody results in release of the detectable entity in a detectable form from the antibody but binding of the antigen to the antibody does not cause such release or causes significantly lower levels of release. Thus antigen in the sample competes with the analogue for the first binding site and so reduces the amount of detectable entity released from the antibody. Thus, the present invention provides an assay for relatively small antigens, such as inhibin, in which competition for a binding site on a multispecific antibody is used to affect the level of detectable entity

detected which is related to the amount of antigen in the sample. Usually, the concentration of antigen and the amount of detected detectable entity will be inversely related.

The term "analogue" as used herein embraces all antigens capable of binding to the same or closely related binding site on the multispecific antibody as the antigen of interest. The analogue may or may not include the same or similar structure as the antigen or a portion thereof. Preferably, the analogue comprises the antigen or a portion thereof linked to a larger entity so that the size of the resulting combination is sufficient to cause the release of bound detectable entity from the antibody.

In one embodiment, the analogue comprises at least a portion of inhibin or a functional equivalent thereof bound to Keyhole Limpet Haemocyanin a widely used antigen about 800 kD in size. Other large molecules may be used as larger entities to form the analogue of inhibin (or any other antigen of interest) such as Bovine Serum Albumin (BSA) although KLH is preferred because it is larger and relatively inexpensive and does not have the cross-reactivity problems of BSA. Typically, any such larger molecule will have a molecular weight exceeding 200kDaltons. Preferably the inhibin comprises at least the β subunit thereof. Functional equivalents of inhibin include recombinant polypeptides having some or all of the biological activity of natural inhibin or portions thereof.

The analogue may comprise a recombinant polypeptide including a portion capable of binding to the binding site or a closely related binding site to which the antigen of interest binds and a biologically redundant portion which is sufficiently large to cause release of the detectable entity as described above.

The antigen may be any small molecule to which a binding site on a multispecific antibody can be directed. Preferably the antigen is inhibin. Thus the invention provides a new and useful assay for inhibin.

The antigen may also be, for example, activin, oligouncleotides, saccharides, growth factors, neurotransmitters, hormones such as progesterone, oestrogen, LH, or FSH. In

principle, polypeptides containing as few as five amino acids may be assayed using the system of the present invention.

The term "multispecific" embraces all antibodies having more than one antigen binding site such as bispecific and trispecific antibodies. Preferably the multispecific antibody is bispecific. The term "antibody" used herein embraces immunoglobulins such IgG, IgA, IgM, IgD and IgE and other proteins or polypeptides having the antigen-binding properties of a naturally-occurring antibody, or antibodies, produced by recombinant DNA technology or any other method.

Preferably, the detectable entity is an enzyme such as glucose oxidase or more preferably β-galactosidase.

The choice of a suitable enzyme is within the scope of the skilled worker in this field.

A preferred diagnostic method in accordance with the invention comprises the steps:

1) Providing the multispecific antibody having bound detectable entity on one binding site thereof;

2) contacting the thus bound multispecific antibody with an analogue of an antigen of interest whereby binding of the analogue to a second binding site on the multispecific antibody results in release of bound detectable entity from the multispecific antibody generating a relatively high detectable signal;

3) contacting the system of step 2) with a sample of interest containing the antigen to be assayed whereby binding of the antigen to the antibody in competition with the analogue results in reduction of the signal level proportional to the amount of antigen of interest in the sample.

The diagnostic method of the invention may be operated on a convenient substrate such as

a dipstick or in homogenous form as a near patient one step test.

In a further preferred embodiment, an enzyme comprising the detectable entity in a first system in accordance with the invention produces substrate for an enzyme comprising the detectable entity in a second system in accordance with the invention. Thus the two enzymes form a cascade. For example, in a diagnostic method for detecting the presence of both α and β subunits of inhibin in a sample, one system in accordance with the invention arranged to detect the β subunit is provided including the enzyme glucose oxidase comprising the detectable entity, and another system in accordance with the invention is provided arranged to detect α subunit in which the enzyme horse radish peroxidase. In the presence of both inhibin subunits, released glucose oxidase produces the substrate for the horse radish peroxidase.

A diagnostic method in accordance with the invention will now be described, by way of example only, with reference to the accompanying drawings Figures 1, 2, 3,4,5 and 6 in which:

Fig. 1 shows a multispecific antibody and associated antigen of interest and analogue;

Fig. 2 a, b and c show schematically the method of the invention;

Fig. 3 shows antigen capture of the β inhibin peptide;

Fig.4 shows antigen capture of glucose oxidase;

Fig. 5 shows partial inhibition of enzyme activity by the antibody; and

Fig. 6 shows a competitve transduction assay.

The bispecific antibody 10 shown in Fig. 1 includes first and second binding sites 12 and 14. First binding site 12 is arranged to bind antigen 16 of interest. Antigen 16 is the β chain of inhibin. Alternatively binding site 12 will bind analogue 18 of the β chain of inhibin 16. Analogue 18 comprises KLH 20 bound to the β chain of inhibin 22 by conventional techniques. Specifically the KLH is succinimide linked to an amino acid inserted at position 23 of the β chain. Second binding site 14 is arranged to bind glucose oxidase 24 whereby its active site 26 is blocked when the enzyme is bound, the active site being unblocked, and therefore active, when the enzyme is released from the antibody.

Antibody 10 was produced using conventional techniques as outlined in our above mentioned co-pending International patent application.

As mentioned above KLH is a relatively large molecule, having a molecular weight of 800 kD. Binding of analogue 18 to bispecific antibody 10, when glucose oxidase 24 is already bound thereto, causes release of the bound glucose oxidase from the antibody in an active form. However, binding of the β chain of inhibin 16 to binding site 12 does not cause release of glucose oxidase bound at binding site 14, presumably because the inhibin does not sterically interact with the bound enzyme. Binding of KLH alone to the antibody will not occur.

The assay method of the present invention is illustrated in Fig. 2 a, b & c.

In the situation illustrated in Fig. 2a, binding of β subunit of inhibin 16 to bispecific antibody 10 does not cause bound glucose oxidase 24 to be released from the antibody 10. Therefore no signal (i.e a colour change) or only background levels of signal, is produced in the presence of the enzyme substrate (e.g TNBT).

In the situation illustrated in Fig. 2b, where antibody 10 having glucose oxidase 24 bound thereto is contacted with the analogue 18 of Fig. 1, glucose oxidase 24 is released on binding of the analogue 18 to binding site. This release causes a high signal.

In contrast in the situation illustrated in Fig. 2c, where β subunit 16 is present in a sample is contacted with the bispecific antibody 10 having bound glucose oxidase 24, in the presence of analogue 18, competition between the β subunit of inhibin 16 and the analogue reduces the amount of bound glucose oxidase which is released leading to a signal which is intermediate in strength between the signals generated in the situations of Figs. 2a and 2b. This detectable reduction in signal is proportioned to the amount of inhibin in the sample.

A Isolation of hybridomas secreting suitable monoclonal antibodiees

Monoclonal antibody-secreting cell lines were prepared recognising a synthetic peptide fragment of the β chain of human inhibin. A polypeptide having the amino acid sequence of residues 82-114 (VPTKLRPMSMLYYDDGQNIIKKDIQNMIVEECG) of the inhibin β sub unit was synthesized using standard peptide synthesis techniques. The resulting human inhibin β chain subunit (the β peptide) was approximately 4kD in molecular weight. The β peptide was conjugated to bovine serum albumin in a 5:8 weight ratio using n-Maleimodobenzoyl-N-hydrosuccinimide ester to give a protein conjugate,β-BSA, of 7kDaltons.

The β peptide was also conjugated to Keyhole Limpet Haemocyanin (KLH) in a 5:8 weight ratio using sulphsuccinimidyl 4 (N-maleimido methyl) cyclohexane-1-carboxylate to give a protein conjugate, β-KLH, of approximately 800kDaltons.

Hybridomas secreting antibodies reognising the inhibin β peptide were prepared by standard methods. The hybridoma cell line E26 secrets a monoclonal antibody reactive with the β peptide. The cell line was isolated from a cell fusion experiment between mouse SP2/0 myeloma cells and splenocytes from a BALB/c female mouse immunized weekly over a four week period with a 10μg per dose of β-KLH conjugate supported with an alum adjuvant. 20μg of β-KLH was given four days prior to the cell fusion experment.

Cell fusion was performed by standard techniques and cultures were screened 17 days later by indirect ELISA. 593 hybridoma cell cultures were obtained, 26 of which were found to secrete antibodies reactive with β-KLH by indirect ELISA. Four of the 26 cultures secreted antibodies which were also reactive with β-BSA and failed to recognise KLH by indirect ELISA indicating that the 4 cell lines secreted antibodies reactive with the inhibin β-peptide. One of these 4 cell lines, the hybridoma E26 was chosen for further study. The cell line secretes a mouse IgG antibody directed against an epitope on the β subunit of inhibin. The antibody does not recognise the α subunit of human inhibin, as indicated by indirect ELISA. The antibody also recognizes the β peptide and β peptide conjugates on Western protein immunoblot analyses.

B Selection of drug resistant clones

Hybridomas secreting suitable monoclonal antibodies were cultured in toxic media to isolate drug resistant clones for bispecific fusoma production.

To induce thioguanine resistance 10^7 E26 hybridoma cells were dispensed into 2x48 well tissue culture plates containing α MEM medium (Stanners CP *et al* (1971) *Nature, New Biol 230,* 52) with 10% (v/v) heat inactivated fetal calf serum, 20%(v/v) conditioned medium from J774 macrophage cell line (*Cancer Res.* (1977), 37, 546) and 5μg per ml of 6-thioguanine (Sigma A4660).

After four weeks, 23 clonal outgrowths were visible. Clones were removed by pipette and subcultured. All 23 cell lines were found to secrete the E26 monoclonal antibody, with no loss of antibody secretion as a result of drug selection. Subclone 3, E26tg3, was chosen for further study, this cell line having a cell doubling time of approximately 18 hours.

C Fusoma Production

Fusomas secreting bispecific antibodies were produced by conventional techniques in cell

fusion events to select those producing bispecific antibodies with an enzyme-reactive arm and a second antibody binding site recognising the antigen of interest.

Fusoma cell line N015 secretes a bispecific immunoglobulin reactive with human inhibin β peptide and glucose oxidase from *Aspergillus niger*. The cell line was isolated from a cell fusion event between hybridoma E26tg3, a thioguanine-resistant cell line secreting antibodies recognising β peptide, and splenocytes from a BALB/c female mouse immunised weekly over a four week period with 10μg per dose of glucose oxidase (Sigma G7141) supported in an alum adjuvant. 20μg of glucose oxidase was given intravenously four days prior to the cell fusion experiment.

Cell fusion was performed by standard techniques and cultures were screened 17 days later by indirect ELISA against glucose oxidase as the target antigen. 371 fusoma cultures were obtained, 84 of which were found to secrete antibody reactive with glucose oxidase by indirect ELISA. 14 cultures secreted immunoglobulin reactive with both β peptide and glucose oxidase as determined by ELISA and Western blot analysis. One of these cell lines, N015 is now described.

The cell line was routinely grown in α MEM and produces approximately 2.0 μg per ml of immunoglobulin in unstirred monolayer culture growth conditions.

Enriched N015 immunoglobulin was isolated by standard affinity chromatography techniques using protein A Sepharose (Sigma P3391). 608μg of immunoglobulin was

isolated from 400 ml of culture medium.

Antigen capture to demonstrate recognition of both human inhibin β peptide and glucose oxidase.

N015 immunoglobulin can be used in an antigen capture ELISA format to detect β peptide and glucose oxidase.

Specific β peptide antigen capture (See Fig. 3).

NO15 immunoglobulin was coated at 10μg per ml in carbonate-bicarbonate buffer, pH 9.6, 50μl per well, in a 96 well flat bottom immunoassay plate (Falcon Cat. No. 3912) overnight incubation at 4°C. Plates were blocked with 100μl per well, 10% (V/V) fetal calf serum in phosphate buffered saline, PBS, 2 hrs at room temperature.

Increasing concentrations of β-KLH conjugate, the specific antigen, and KLH protein, a non-specific antigen were loaded in 50μl volumes in duplicate, from 0-20μg per ml and incubated for 1 hour at 100m temperature. Both β-KLH and KLH antigens were substituted in the approximate ratio of 3 biotin molecules per protein molecule. Stock β KLH biotin was 0.33 mg per ml for dilution, and stock KLH biotin 1 mg per ml for dilution.

Wells were washed twice with 200μl PBS 0.5% (v/v) Tween 20, PBS Tween, and then bound antigen was detected by addition of Avidin-enzyme conjugate and developing for

bound enzyme. Wells were incubated with 50μl of 1:500 dilution Avidin-alkaline phosphatase (1mg per ml stock in PBS: Sigma A2527) in PBS with 0.5% (w/v) BSA (Sigma A7888) for 30 minutes at 4°C. Wells were washed three times in PBS Tween and then presence of alkaline phosphatase was detected by substrate conversion of para-nitrophenyl phosphate (Sigma 104-105E). Briefly, 50μl per well of the substrate was added at 1mg per ml in 1M diethanolamine buffer, pH9.8, comprising 97ml diethanolamine, 800ml water, 100mg magnesium chloride hexahydrate, with 1M hydrochloric acid added until the ph is 9.8, the volume then made up to 1 litre with water. Substrate conversion by enzyme to product was detected by measurement of optical density of 410mn.

Specific recognition of the β peptide by NO15 bispecific antibody is illustrated in Figure 3. Significant capture of the β peptide KLH conjugate is seen from 1μg per ml antigen concentration. In contrast, the non-specific antigen, KLH is not recognised and captured by the NO15 bispecfic antibody.

Glucose Oxidase antigen capture (See Fig. 4).

Concentrations of NO15, from 0 to 10μg per ml were coated into wells of a 96 well flat immunoassay plate using the carbonate-bicarbonate buffer method as above. Plates were then blocked ready for use.

Wells were incubated with 50μl per well glucose oxidase, 10μg per ml in PBS with 0.5% (w/v) BSA, the PBS/BSA buffer. The enzyme solution was incubated for 1 hour at 4°C and

the plates then washed three times with PBS Tween. Bound enzyme was then detected by addition of the enzyme substrate and subsequent conversion to product. Substrate conversion by enzyme was measured by optical density at 630nm.

Briefly, the glucose oxidase substrate was 0.82mM 3,3'-(3,3'-dimethoxy-4,4' biphenylylene)-bio-(2,5,p-nitrophenyl-2H-tetrazolium chloride, TNBT, (Sigma T4000)with 6.7mg per ml β-D-glucose (Sigma G5250) and 0.0167 mg per ml phenazine methosulphate (Sigma P9625) in 50 mM Tris buffer pH 8.4. The substrate was prepared immediately before use and the developing microtitre plates stored in the dark.

Specific capture of the glucose oxidase enzyme by NO15 bispecific antibody is illustrated in Figure 4. An antibody concentration of 0.625μg per ml is sufficient to capture glucose oxidase and at increasing antibody capture concentrations, enzyme activity is detected but at a reduced level. This suggests that NO15 antibody partially inhibits the activity of glucose oxidase, perhaps by recognition of an epitope near the active site of the enzyme.

NO15 antibody partially inhibits the activity of glucose oxidase (See Fig. 5).

25 μl aliquots of NO15 immunoglobulin at 10μg per ml were added to 25μl volumes of glucose oxidase solution in PBS/BSA buffer, concentrations from 0 to 10μg per ml enzyme, in wells of a 96 well microlitre plate. Equivalent control concentrations of glucose oxidase were prepared in 50μl volumes, in PBS/BSA buffer. The enzyme and antibody solution and

the enzyme controls were incubated for 30 minutes at room temperature. 50μl per well of glucose oxidase substrate solution were then added to measure residual glucose oxidase activity. Conversion to product was measured by optical density at 630nm.

Partial inhibition of glucose oxidase activity by NO15 bispecific antibody is illustrated in Figure 5. At all concentrations of enzyme, a concentration of 10μg per ml NO15 antibody is found to reduce substrate conversion to product.

Demonstration of competitive antibody-mediated signal transduction (see Fig. 6).

Competitive transducing antibody activity has been demonstrated with NO15 antibody. Immobilized NO15 immunoglobulin was loaded with glucose oxidase (the "detectable entity") to form a transducing antibody-enzyme complex. The complex was then exposed to a standard concentration of the high molecular weight specific β antigen conjugate β-KLH, molecular weight 800kDaltons (the "analogue") or equivalent concentration of non-specific high molecular weight protein KLH, molecular weight 800kDaltons, with inclusion in both experimental sets of varying concentrations of the low molecular weight competitor, the β inhibin peptide of 3kDaltons (the "antigen"), specifically recognised by the NO15 bispecific antibody. Supernatant was then developed to detect for the presence of released glucose oxidase by enzymatic conversion of substrate to product.

The two events associated with competitive antibody-mediated transduction are illustrated in Figure 6. First, enzyme,the detectable entity, release only occurs at significant levels in

the presence of the specific antigen of sufficiently high molecular weight to cause enzyme release, namely the conjugate between β peptide and KLH, β KLH, the analogue. Although, β inhibin peptide ,the antigen, is applied in the presence of the non specific antigen KLH, there is no significant release of enzyme, the peptide being too small to induce antibody-mediated signal transduction by NO15 antibody.

Second, specific competitive antibody-mediated transduction is observed in the presence of the antigen and analogue of differing molecular weight. At low concentrations of β peptide,the antigen, below 2.5μg per ml, transduction occurs in the presence of the standard concentration of β KLH,the analogue, 10μg per ml. However at high concentrations of the small antigen, competition for the antibody binding site of NO15 is seen. Enzyme release, mediated by signal transduction with the high molecular weight specific β-KLH, analogue,is reduced as the concentration of the low molecular weight specific antigen is increased. Concentration of the small antigen β peptide, of 4kDaltons molecular weight is therefore measured by the decrease in enzyme release generated by the competitive antibody signal transduction event.

Briefly, NO15 immunoglobulin was coated at 5μg per well in ELISA coating buffer as above and plates were blocked ready for use. Wells were then incubated with 50μl of 10μg per ml of glucose oxidase in PBS/BSA buffer. The plates were incubated for 1 hour at room temperature. Wells were then washed twice with 200μl of PBS Tween to remove unbound enzyme from the immobilized transducing antibody complex.

A series of concentrations of the specific low molecular weight β peptide were prepared from 0-40μg per ml, all protein dilutions being performed in PBS/BSA buffer. An equal volume of the specific high molecular weight competitor analogue, β KLH at 20μg per ml or the non specific protein KLH at 20μg per ml was then added to the β peptide. 50μl of the competitor mixtures were then added to the wells containing the preformed transduction complex. For the β KLH/β peptide competition, concentrations under test were from 10μg per ml β KLH/0μg per ml β peptide to 10μg per ml β KLH/20μg per ml β peptide. For the control experiment, concentrations under test were from 10μg per ml KLH/0μg per ml β peptide to 10μg per ml KLH/20μg per ml β peptide. Plates were incubated for 30 minutes at room temperature. The supernatants were then removed from each well and tested for the presence of released enzyme by addition of 50μl per well glucose oxidase substrate solution. Enzymatic conversion of substrate to product was monitored by measuring optical density at 630nm after one hour incubation in the dark at room temperature.

Competitive antibody transduction is demonstrated in Figure 6. In the presence of increasing concentrations of the β peptide, specific enzyme release, mediated by recognition of β KLH and transduction by the NO15 bispecific antibody, is reduced. Concentration of the molecular analyte is directly indicated by the degree of reduction in enzyme activity.

Bispecific antibody NO15 has been deposited under the Budapest Treaty at the European Collection of Animal Cell Cultures, Porton Down under the accesion number 94080534. Thus the invention provides an effective assay for relatively small molecules such as

inhibin.

CLAIMS

1 A diagnostic method for determining the presence, or absence of an antigen in a sample, the method comprising contacting the sample with a multispecific antibody having first and second binding sites for binding the antigen and a detectable entity respectively, binding of the detectable entity to the antibody inactivating the detectable entity in the presence of an analogue of the antigen, wherein binding of the analogue of the antigen to the antibody results in release of the detectable entity in a detectable form from the antibody but binding of the antigen to the antibody does not cause such release or causes significantly lower levels of release.

2 A diagnostic method comprising:

1) providing a multispecific antibody having bound detectable entity on one binding site thereof;

2) contacting the thus bound multispecific antibody with an analogue of an antigen of interest, whereby binding of the analogue to a second binding site on the multispecific antibody results in release of bound detectable entity from the multispecific antibody generating a relatively high detectable signal;

3) contacting the system of step 2) with a sample of interest containing the antigen to be assayed whereby binding of the antigen to the antibody in

competition with the analogue results in reduction of the signal level proportional to the amount of antigen of interest in the sample.

3 A diagnostic method according to claim 1 or 2 in which antigen in the sample competes with the analogue for the first binding site and so reduces the amount of detectable entity released from the antibody.

4 A diagnostic method according to claim 1, 2 or 3 in which the antigen is inhibin, a portion thereof or a functional equivalent thereof.

5 A diagnostic method according to any preceding claim in which the concentration of antigen and the amount of detected detectable entity are inversely related.

6 A diagnostic method according to any preceding claim in which the analogue comprises the antigen or a portion thereof or a functional equivalent thereof linked to a larger entity so that the size of the resulting combination of the antigen or portion thereof and the larger entity is sufficient to cause the release of bound detectable entity from the antibody.

7 A diagnostic method according to claim 4 in which the analogue comprises at least a portion of inhibin or a functional equivalent thereof bound to Keyhole Limpet Haemocyanin (KLH).

8 A diagnostic method according to claim 7 in which the inhibin comprises at least the β subunit thereof or a functional equivalent thereof.

9 A diagnostic method according to any one of claims 4 to 8 in which the functional equivalent of inhibin comprises a recombinant polypeptide having some or all of the biological activity of natural inhibin or a portion thereof.

10 A diagnostic method according to any preceding claim in which, the analogue comprises a recombinant polypeptide including a portion capable of binding to the binding site or a closely related binding site to which the antigen of interest binds and a biologically redundant portion which is sufficiently large to cause release of the detectable entity as described above.

11 A diagnostic method according to any one of claims 1,2,3,5,6, or 10 in which the antigen is selected from activin, oligouncleotides, saccharides, growth factors, neurotransmitters, hormones including progesterone, oestrogen, LH, or FSH.

12 A diagnostic method according to any preceding claim in which the antigen comprises a polypeptide comprising five or more amino acids.

13 A diagnostic method according to any preceding claim in which the multispecific antibody is a bispecific antibody.

14 A diagnostic method according to any preceding claim in which the detectable entity is an enzyme.

15 A diagnostic method according to any preceding claim arranged to be operated on a substrate

16 A diagnostic method according to claim 15 in which the substrate is a biosensor.

17 A diagnostic method according to claim 15 in which the substrate is a dipstick.

18 A diagnostic method according to any preceding claim arranged to be operated in homogenous system.

19. A diagnostic method according to any preceding claim in which the antibody is NO15 deposited under the Budapest Treaty on 5th August 1994 at the European Collection of Animal Cell Cultures, Porton Down under accession no. 94080534.

20. A diagnostic method according to any preceding claim in which an enzyme comprising the detectable entity in a first system arranged to operate a diagnostic method in accordance with the invention produces the substrate for an enzyme comprising the detectable entity in a second system arranged to operate a second diagnostic method in accordance with the invention .

21 A diagnostic method according to claim 20 for detecting the presence of both α and β subunits of inhibin in a sample, in which a first system in accordance with the invention arranged to detect the β subunit is provided in which glucose oxidase comprises the detectable entity, and a second system in accordance with the invention is provided arranged to detect α subunit in which horse radish peroxidase comprises the detectable entity, whereby, in the presence of both inhibin subunits, released glucose oxidase from the first system produces the substrate for the horse radish peroxidase of the second system.

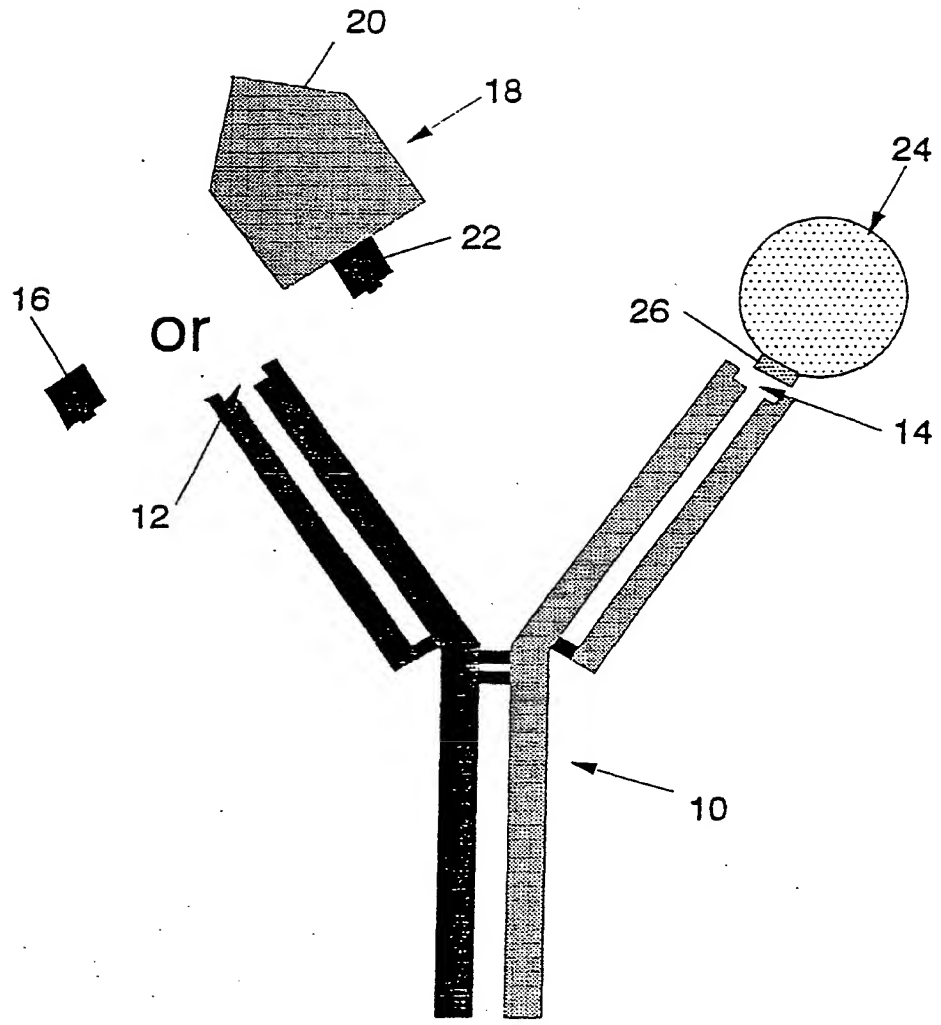


Figure 1

Figure 2

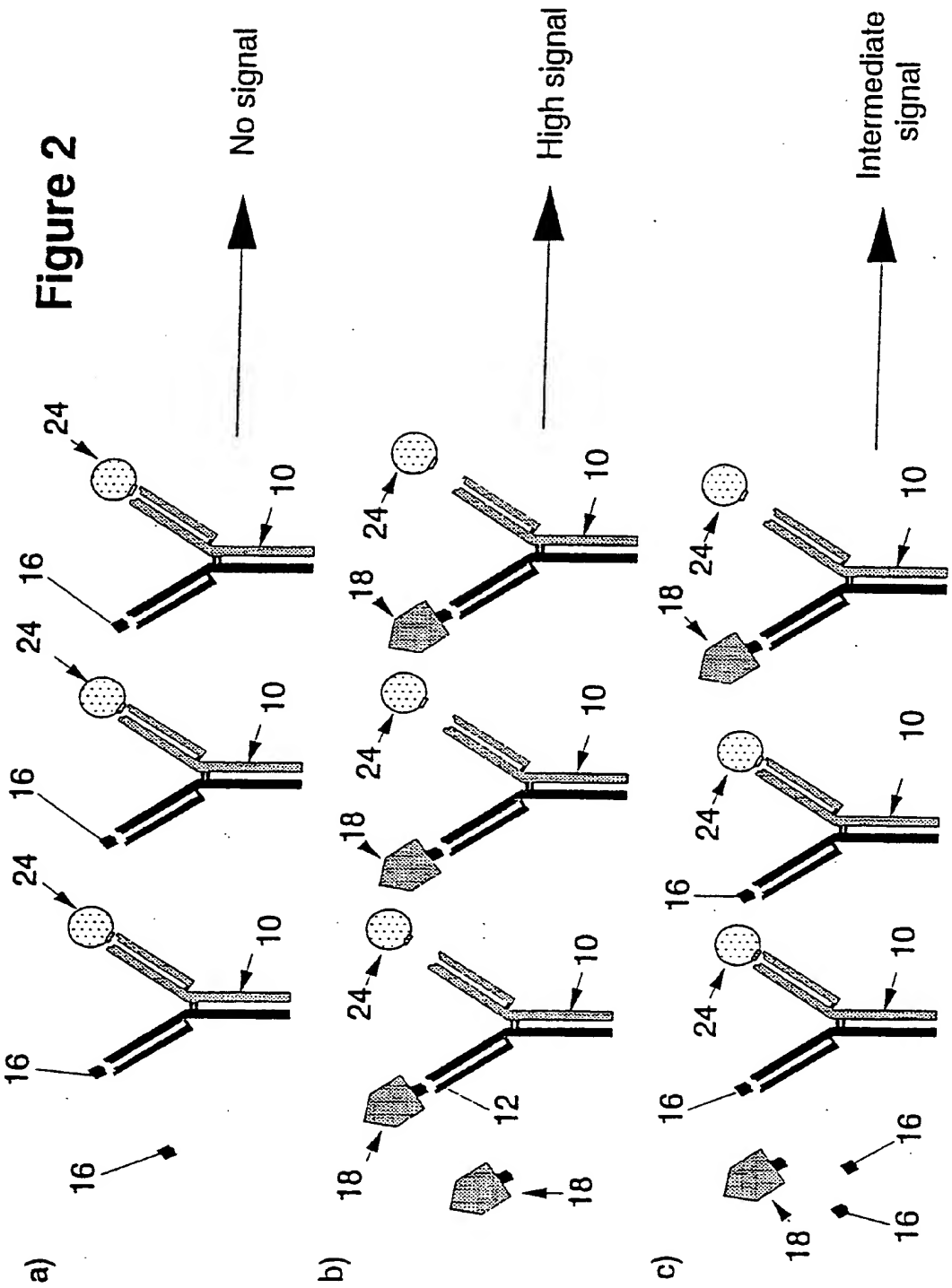


Figure 3

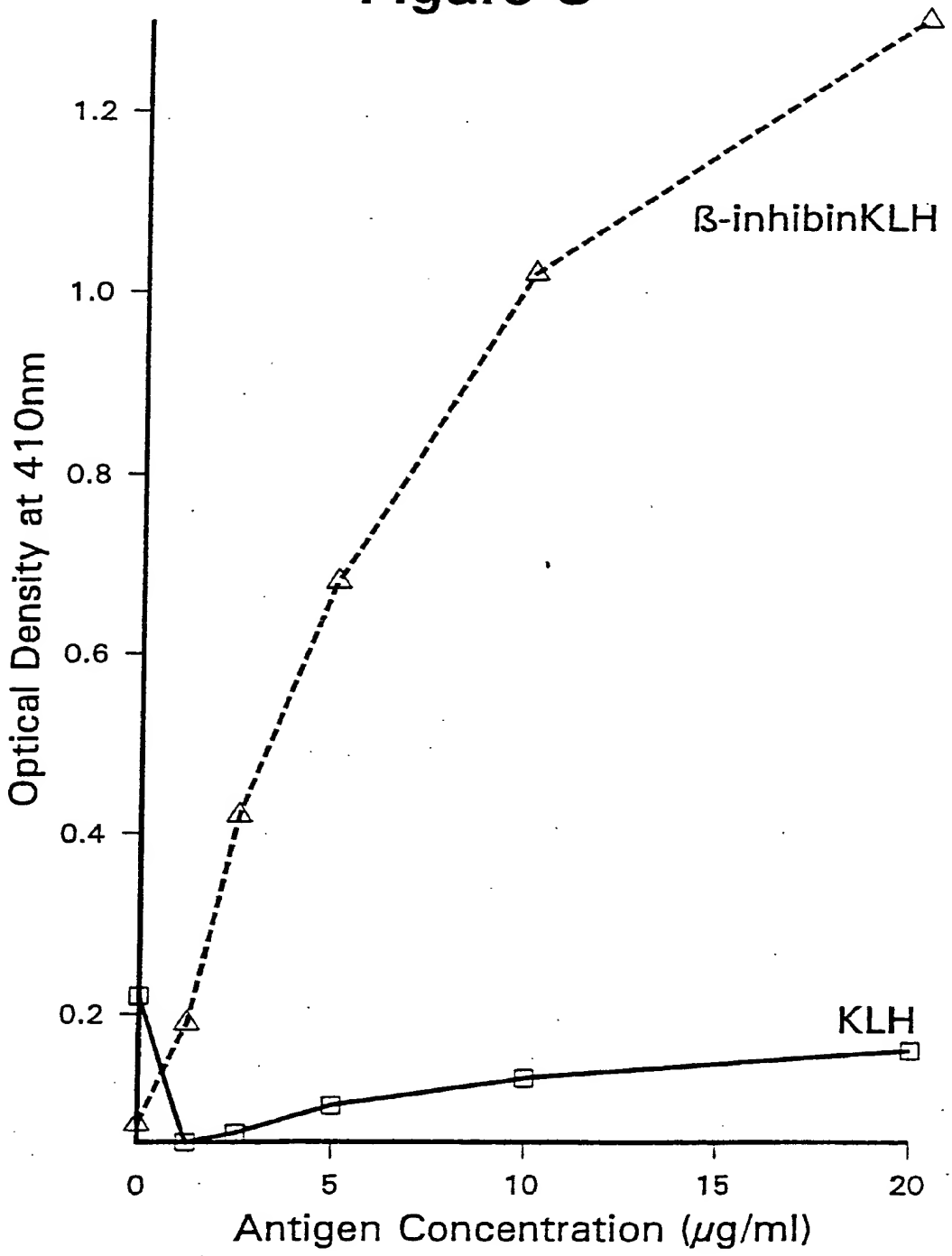


Figure 4

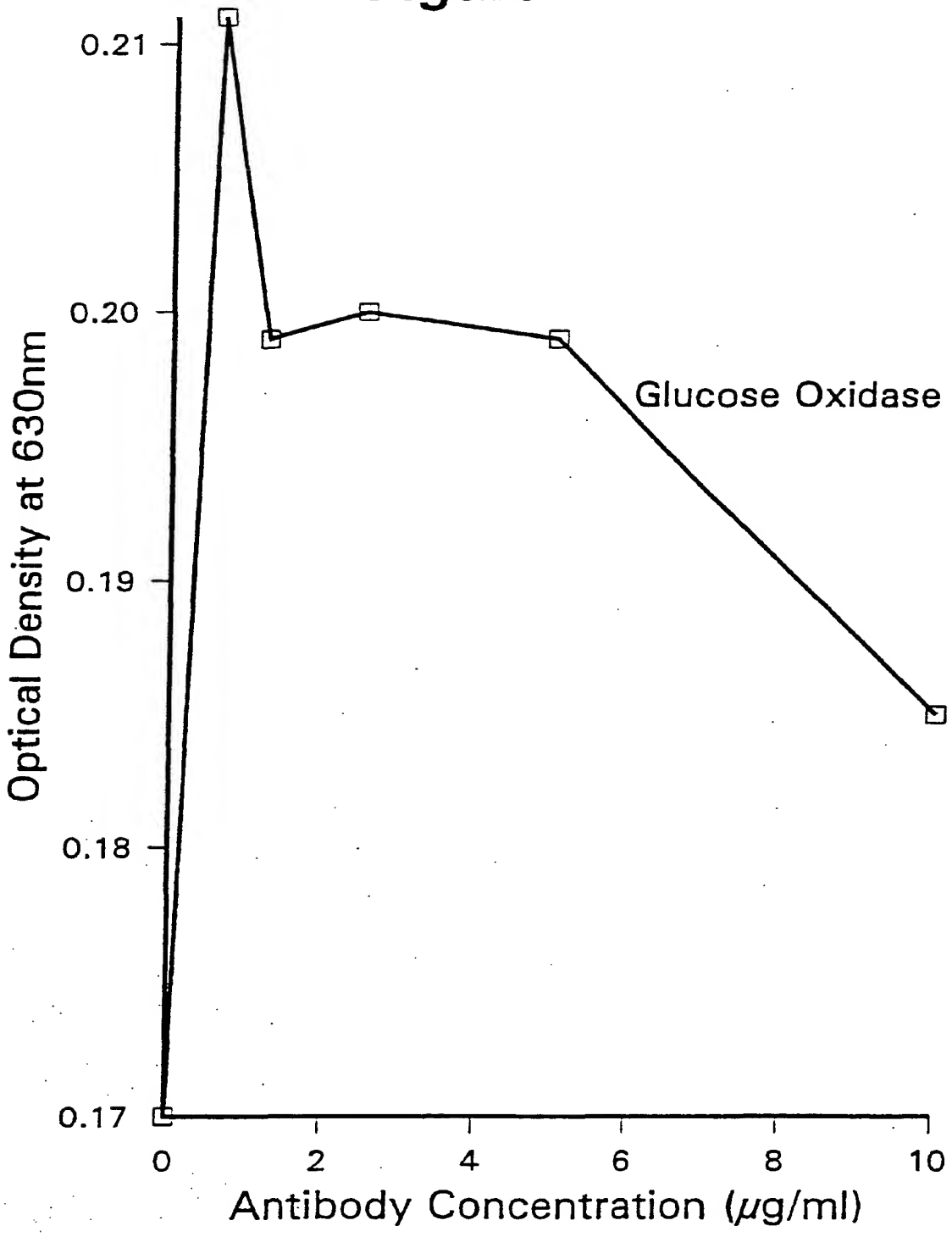


Figure 5

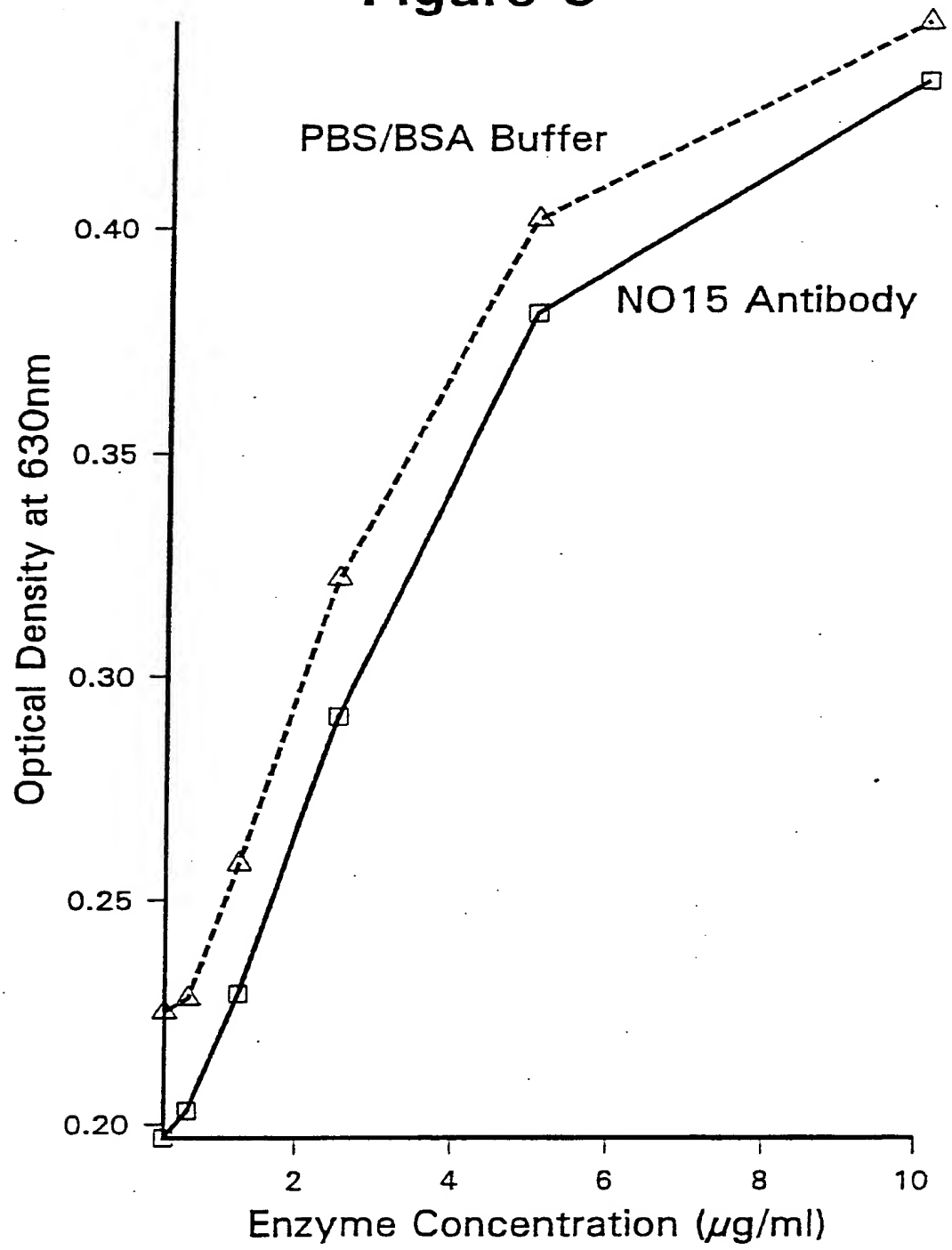
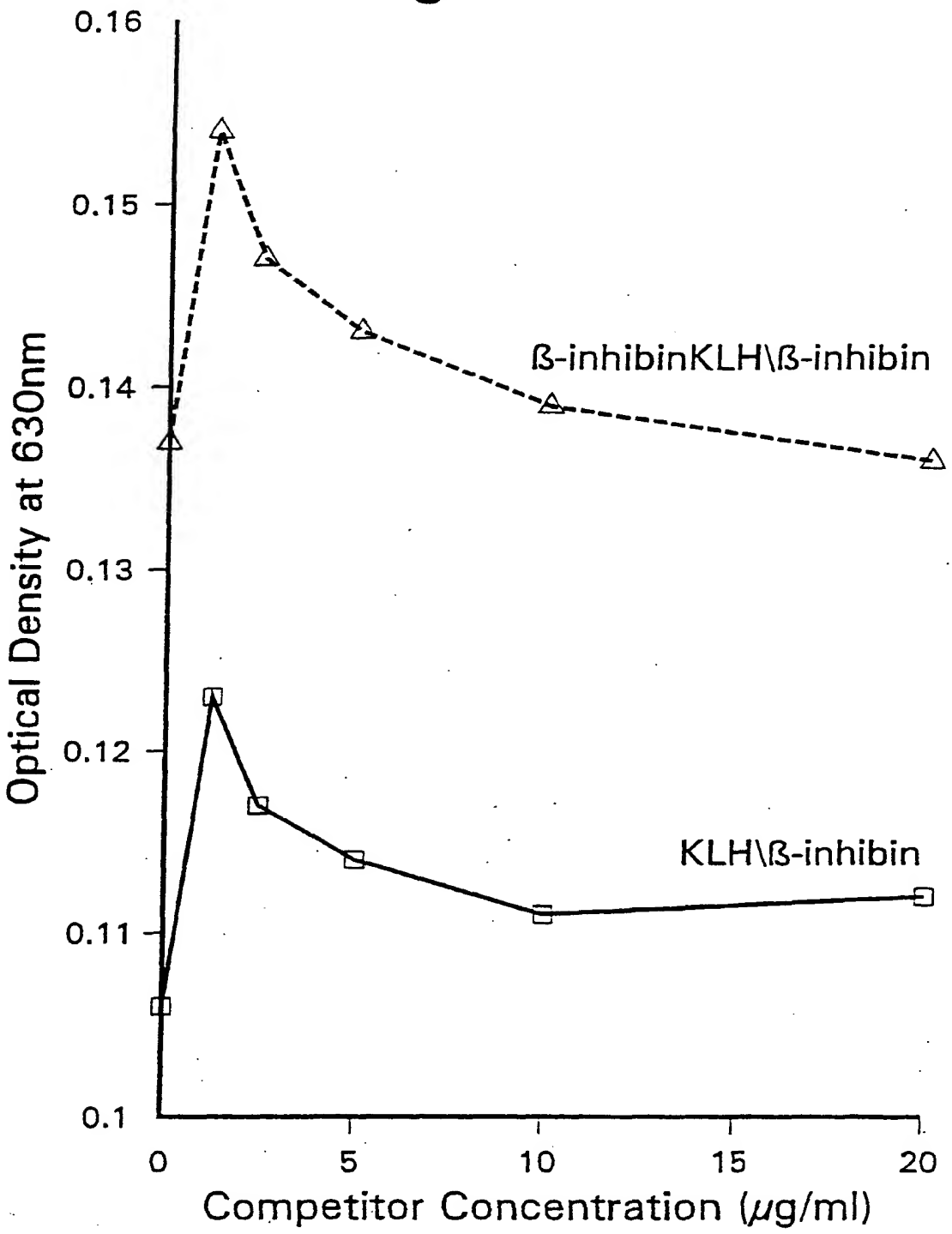


Figure 6



INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/GB 94/01739

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/542 G01N33/74 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP,A,0 511 011 (SURFACE ACTIVE LIMITED) 28 October 1992 cited in the application see the whole document	1-3,5-7, 12-18,20 4,8-11, 19,21
Y	WO,A,83 03679 (HYBRITECH INCORPORATED) 27 October 1983 see the whole document	1-21
Y	WO,A,90 07714 (APPLIED RESEARCH SYSTEMS ARS HOLDING NV.) 12 July 1990 see the whole document	1-21
Y	US,A,4 864 019 (W. W. VALE ET AL.) 5 September 1989 see the whole document	1-21

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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1994	21. 12. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Griffith, G

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Inter. nal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	EP,A,0 096 463 (E. I. DU PONT DE NEMOURS AND COMPANY) 21 December 1983 see the whole document -----	1-21

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